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Syntheses and biological investigations of kirkamide and oseltamivir hybrid derivatives

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ABSTRACT

The C₇N-aminocyclitol kirkamide was recently isolated from the plant obligate symbiont *Candidatus Burkholderia kirkii* and was hypothesized to be beneficial to the plant host due to its cytotoxic activity against insects and arthropods. To study its mechanism of action and inspired by its structural similarity with *N*-acetylglucosamine (GlcNAc) and oseltamivir, kirkamide-oseltamivir hybrid derivatives were synthesized and investigated for their biological activity. Interestingly, kirkamide analogues were reasonably potent against a known bacterial neuraminidase.

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1. Introduction

Natural products remain a prime source of inspiration for the development of new lead structures in drug discovery [1,2]. Recently, two novel natural products, kirkamide (1) and streptol glucoside (2) have been isolated from an obligate symbiosis system composed of the plant *Psychotria kirkii* and the bacterium *Candidatus Burkholderia kirkii* [3,4]. Synthetic routes towards accessing these natural products and derivatives have been already identified in the literature [3,5,6]. The discovery of kirkamide (1) was guided by the genome-based prediction of 2-epi-5-epi-valiolone [7,8], which is known to be involved in the biosynthesis of the C₇N aminocyclitol family, a rich source of bioactive compounds [9–13]. The cytotoxicity of kirkamide (1) was evaluated against several organisms, such as insects and arthropods [3]. The structural similarity between *N*-acetylglucosamine (3, GlcNAc), oseltamivir (4) [14], and kirkamide (1) suggested that the natural aminocyclitol 1 could act as a glycoside hydrolase (glycosidase) inhibitor (Fig. 1).

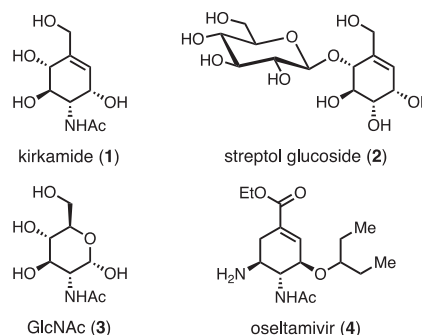


Fig. 1. Constitution and configuration of kirkamide (1), streptol glucoside (2), *N*-acetylglucosamine (3, GlcNAc), and oseltamivir (4).

The aim of this study was to (a) prepare hybrid compounds between kirkamide (1) and oseltamivir (4), (b) investigate plausible enzyme targets, and (c) evaluate the potential of oxidation at the methylene allylic position of kirkamide (1) under biologically relevant conditions. In order to reach these goals, we employed a divergent synthetic route allowing for late stage modification of a synthetic intermediate.

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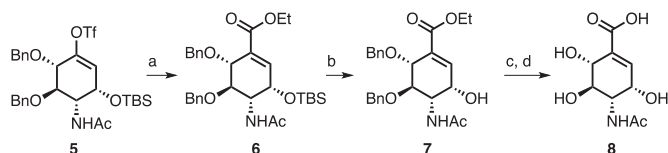
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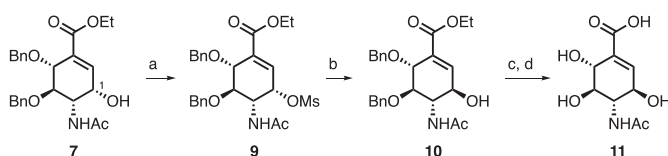
2. Results and discussions

To achieve our aims, triflate **5** [3] was selected as the key intermediate for the synthesis of all compounds (Scheme 1). Evaluation of different electrophilic precursors for the subsequent carbonylation, both by us [3,6] and others [5], all provided evidence for the superiority of enol triflates over enol halogenides. Consequently, using the standard Pd-mediated carbonylation/esterification conditions, the ethyl ester **6** was synthesized in good yield (68%) and smoothly Si-protected using TBAF in THF to obtain the alcohol **7**. The benzyl deprotection was found to be challenging: In contrast to the hydroxymethylene derivative in the kirkamide synthesis [3], the ester **7** proved to be recalcitrant under classical Birch conditions, eventually leading to the decomposition of the starting material. After screening of several Lewis acids, only BCl_3 in CH_2Cl_2 delivered the triol **8** in low yield. Pleasingly, the addition of pentamethylbenzene [15] to this reaction mixture significantly increased the yield; and the ester was subsequently saponified with KOH solution to obtain the kirkamide carboxylic acid derivative **8**.



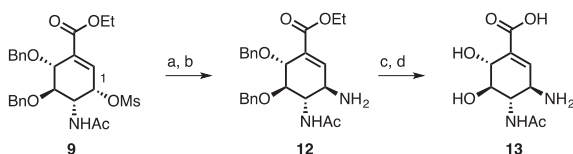
Scheme 1. Synthesis of the carboxylic acid derivative **8**. a) $\text{Pd}(\text{OAc})_2$, PPh_3 , CO , NEt_3 , 68%; b) TBAF, 74%; c) BCl_3 , $\text{Me}_5\text{C}_6\text{H}$, CH_2Cl_2 ; d) KOH, 53% over 2 steps.

The free hydroxyl group of the available ester intermediate **7** (Scheme 2) was activated with mesyl chloride to obtain mesylate **9** (71%, purified by flash chromatography on silica) and its configuration at position 1 was subsequently inverted, using KNO_2 , to the C(1) epimer **10** [16]. Using the optimized deprotection conditions developed earlier, the second kirkamide derivative **11** was smoothly synthesized via debenzoylation.



Scheme 2. Synthesis of the epimer at position 1. a) MsCl , DMAP, NEt_3 , 71%; b) KNO_2 , 18-crown-6, 57%; c) BCl_3 , $\text{Me}_5\text{C}_6\text{H}$, CH_2Cl_2 ; d) KOH, 52% over 2 steps.

Taking advantage of the mesyl intermediate **9** (Scheme 3), the amine **12** was synthesized in two steps composed of a $\text{S}_\text{N}2$ type reaction with azide following by a Staudinger reaction using PPh_3 as reductant. We were pleased by the good yield of this two-step procedure and the full deprotection was performed to obtain the amine kirkamide derivative **13**.



Scheme 3. Synthesis of the amine derivative **13**. a) NaN_3 ; b) PPh_3 , 62% over 2 steps; c) BCl_3 , $\text{Me}_5\text{C}_6\text{H}$, CH_2Cl_2 ; d) KOH, 94% over 2 steps.

Synthetic kirkamide **1** and its derivatives **8**, **11**, and **13** were evaluated as potential inhibitors of various *N*-acetyl-D-

Table 1

Inhibitory activity (IC_{50} , μM) of kirkamide (**1**) and derivatives.

	1	8	11	13
Human Hexosaminidase B (HexB)	689	>1000	>1000	280
NagZ from <i>E. coli</i> (EcNagZ)	624	912	>1000	347
Human NAGLU	240	595	>1000	881
Jack bean β -hexosaminidase	720	>1000	776	130
Chitinase from <i>S. griseus</i>	>1000	>1000	>1000	>1000
Human Chitotriosidase	>1000	>1000	>1000	>1000
BbLNBase	830	>1000	>1000	764
Neuraminidase from <i>C. perfringens</i>	>500	>500	96	75

glucosaminidases due to their structural similarity to the valienamine class of compounds and oseltamivir (**4**) [14,17,18]. We used a panel of enzymes (HexB, EcNagZ, NAGLU, Jack bean β -hexosaminidase, chitinase from *Streptomyces griseus* (*S. griseus*), human chitotriosidase and BbLNBase) that are known *N*-acetyl-D-glucosaminidases (Table 1). Interestingly kirkamide (**1**) was found to be weakly active against human NAGLU ($\text{IC}_{50} = 240 \mu\text{M}$) and was modestly active against the other *N*-acetylglucosaminidases that process single GlcNAc residues. From all the hybrid derivatives, **13** possessing an amine functional group at the C(1) position exhibits the highest inhibition for HexB, EcNagZ, Jack bean β -hexosaminidase and BbLNBase. In comparison, kirkamide (**1**) displays generally better activity than the oxidized derivatives **8** and **11**, and surprisingly, replacement of the OH group by the basic amine (\rightarrow **13**) resulted in better activity against three of the enzymes tested (HexB, EcNagZ, NAGLU).

In addition, we assessed kirkamide (**1**) and the synthesized derivatives against a neuraminidase from *Clostridium perfringens* (*C. perfringens*) due to the structural similarity of **1** with oseltamivir (**4**). Interestingly compounds **11** and **13** possess potency towards the enzyme even though they lack the 3-pentyl sidechain of **4**, which is generally required for good activity against bacterial and viral neuraminidases [14,19]. Interestingly, the des-3-pentyl derivative of oseltamivir (**4**) also displayed only micromolar activity and the introduction of the 3-pentyl chain boosted activity by three orders of magnitude [14].

Another possible mode of action, and potentially more interesting, is that kirkamide (**1**) acts as a *N*-acetylglucosamine surrogate and is incorporated into the UDP-GlcNAc (**14**) salvage pathway found within eukaryotes [20]. Kirkamide (**1**) would be metabolized into UDP-kirkamide (**15**) (Fig. 2) and potentially act as an inhibitor of O-GlcNAc transferases, critical enzymes in chitin biosynthesis [21], a common polysaccharide found in arthropods and insects [22]. Indeed, this type of incorporation of unnatural GlcNAc-based compounds has literature precedent [23–25].

We decided to use a model system taking advantage of the O-GlcNAc modification which is a post-translational modification found in eukaryotes with the enzyme facilitating this modification, O-GlcNAc transferase (OGT) using UDP-GlcNAc (**14**) as its substrate. Utilizing a per-acetylated version of kirkamide **16**, prepared by treatment of kirkamide (**1**) with excess acetic anhydride, we treated

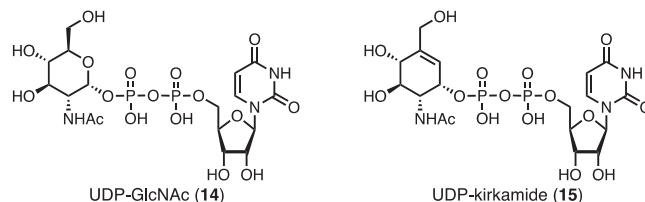


Fig. 2. Comparison between the structure of UDP-GlcNAc (**14**) and UDP-kirkamide (**15**).

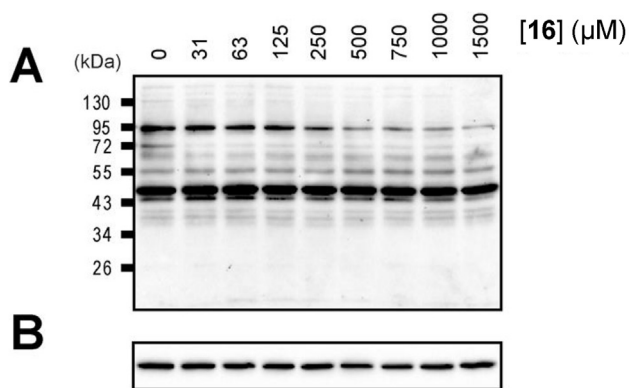


Fig. 3. Incubation of COS-7 cells with **16** causes no clear decrease in cellular levels of O-GlcNAc-modified proteins. Western blot analysis of proteins from COS-7 cells cultured for 24 h in the presence or absence of **16**. A) Western blot analysis of cellular O-GlcNAc levels using mouse anti-O-GlcNAc followed by goat anti-mouse HRP conjugate. B) Western blot of samples loaded in treated with mouse anti- β -actin followed by a rabbit anti-mouse HRP conjugate reveals equivalent β -actin levels in each sample.

the model cell line COS-7 using various concentrations of **16**. A similar strategy has been applied for other systems and showed an increase in the uptake of carbohydrate-based compounds into cells [26]. Unfortunately, we found that there was no clear decrease in O-GlcNAc modified proteins even at high concentrations of **16** (Fig. 3) and concentrations of **16** above 1.5 mM were toxic. This result does not rule out that kirkamide (**1**) is converted to UDP-kirkamide (**15**) to act as a protective compound against organisms affecting *Psychotria-Burkholderia* leaf nodule symbiosis but further studies are required to evaluate this hypothesis.

Finally, we have investigated the possibility that the oxidized kirkamide derivative **8** is present *in planta*, for example as a by-product by oxidation of the allylic methylene group of **1**. In addition, we wanted to test if plant extracts are able to mediate the oxidation of **1** to **8**. However, under several conditions evaluated, no oxidation products such as **8** could be detected. While this does not rule out that no oxidation takes place *in planta*, these observations together with the decreased biological activity against some of the enzymes tested in this study, render the carboxylate **8** less likely to be an active material in the *Burkholderia*/*Psychotria* symbiosis.

3. Conclusions

In conclusion, the mechanism of action of kirkamide (**1**) was investigated by testing its activity against enzymes involved in the metabolism of structurally related compounds. Surprisingly, kirkamide (**1**) did not exhibit a strong inhibition against these potential biological targets. Furthermore, hybrid oseltamivir-kirkamide derivatives were synthesized using a divergent approach and the compounds were evaluated. Interestingly some of the derivatives showed a low micromolar activity against neuraminidases which could result in the development of kirkamide-based compounds towards this important class of enzyme. Overall, based on this and previously published data and the strong phenotype activity of kirkamide (**1**) on insects and arthropods, we hypothesize that the natural product target(s) is/are related to other metabolic pathways that await discovery.

4. Experimental section

4.1. General information, materials and equipment

All chemicals were purchased from Acros, Fluka or Sigma-

Aldrich and were used without further purification (except for Et_3N , which was freshly distilled before use). All reactions were carried out in heat gun-dried glassware (unless aqueous reagents were used) and reactions involving air sensitive compounds were performed under an argon or nitrogen atmosphere. Solvents applied for chemical transformations were either puriss quality or HPLC grade solvents, which had been dried by filtration through activated aluminum oxide under nitrogen (H_2O content <10 ppm, *Karl-Fischer* titration). For work-up and purification solvents were distilled from technical grade. All synthetic transformations were monitored either by thin layer chromatography (TLC) or ^1H NMR spectroscopy. Yields refer to purified, dried and spectroscopically pure compounds. TLC was performed on Merck silica gel 60 F254 plates (0.25 mm thickness) pre-coated with a fluorescent indicator. Concentration under reduced pressure was performed by rotary evaporation at 40°C . Flash chromatography was performed using silica gel 60 (230–400 mesh) from Sigma-Aldrich with a forced flow eluent at 0.1–0.3 bar pressure. Preparative HPLC was carried out on a Shimadzu HPLC system and a fraction collector. All ^1H and ^{13}C NMR spectra were recorded using a Bruker Avance 400 MHz, 500 MHz, 600 MHz (^1H) & 101 MHz or 126 MHz (^{13}C) spectrometer at RT (unless otherwise stated). Chemical shifts (δ -values) are reported in ppm, spectra were calibrated relative to the residual proton chemical shifts (CHCl_3 , $\delta = 7.26$; D_2O , $\delta = 4.79$; $\text{DMSO}-d_6$, $\delta = 2.50$) and the residual carbon chemical shifts (CDCl_3 , $\delta = 77.16$, $\text{DMSO}-d_6$, $\delta = 39.52$) of the solvents, multiplicity is reported as follows: s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet or unresolved and coupling constant J in Hz. IR spectra were recorded on a PerkinElmer SpectrumTwo ATR-FTIR. The absorptions are reported in cm^{-1} . All mass spectra (HRMS-ESI) were recorded by the Mass Spectrometric Service of the University of Zürich on a QExactive instrument (Thermo Fisher Scientific, Bremen, Germany) equipped with a heated electrospray (ESI) ionization source and connected to a Dionex Ultimate 3000 UHPLC system. Melting points (M.p.) were determined using a Büchi B-545 apparatus in open capillaries and are uncorrected. Optical rotations $[\alpha]_D^{25}$ were measured at the sodium D line using a 1 mL cell with a 1 dm or 0.1 dm path length on a Jasco P-2000 digital polarimeter and the concentration c is given in g/100 mL CHCl_3 .

4.2. Biological assay

4.2.1. Kinetic analysis of kirkamide (**1**)

Assays against the enzymes were carried out in triplicate at 37°C for 30 min using a stopped assay procedure in which the enzymatic reactions were quenched by the addition of a 4-fold excess of quenching buffer (200 mM glycine, pH 10.75). Assays against HexB were conducted in buffer (50 mM sodium citrate, 100 mM NaCl, pH 4.25), jack bean β -hexosaminidase (50 mM sodium citrate, 100 mM NaCl, 0.1% BSA w/v, pH 5.0) and *Ec*NagZ (50 mM Hepes, 500 mM NaCl, pH 7.1) using 4-nitrophenyl N -acetyl- β -D-glucosaminide as substrate. Assays against human chitotriosidase were conducted in buffer (100 mM sodium citrate, 200 mM sodium phosphate, pH 5.2), Chitinase from *S. griseus* (50 mM sodium citrate, 50 mM sodium phosphate buffer, pH 5.0) using 4-methylumbelliferyl β -D- N,N,N' -triacetylchitotriose as substrate. Assays against *Bifidobacterium bifidum* lacto- N -biosidase (*Bb*LNBase) were conducted in buffer (50 mM sodium citrate, 50 mM sodium phosphate, pH 4.5) using 4-methylumbelliferyl 3-O- β -D-galactopyranosyl- N -acetyl- β -D-glucosaminide as substrate. For NAGLU, assays were performed in sodium acetate buffer (100 mM, pH 4.3), containing bovine serum albumin (0.5 mg mL^{-1}) using 4-nitrophenyl N -acetyl- α -D-glucosaminide as substrate.

Assays against the neuraminidase from *C. perfringens* were conducted in buffer (50 mM sodium citrate, 50 mM sodium phosphate, pH 5.0) using 2'-(4-methylumbelliferyl)- α -D-N-acetylneuraminic acid as substrate. Output of activity after enzymatic reactions were quenched was recorded using a Spectramax spectrometer (Molecular Devices) at 400 nm for those using 4-nitrophenolate as an output and a Varian CARY Eclipse Fluorescence Spectrophotometer 96-well plate system using excitation and emission wavelengths of 368 and 450 nm respectively, with 5 mm slit openings for those using 4-methylumbelliferonate as an output. Assays contained substrate at the previously determined K_m value of the substrate for the enzyme, and the enzyme was at a concentration of 20 nM.

4.2.2. Cell culture and inhibition

COS-7 cells (Sigma Aldrich ECACC) were cultured in Dulbecco's modified Eagle's medium (DMEM) containing high glucose and no glutamine (Gibco) supplemented with 5% fetal bovine serum (Bovogen) and 2 mM Glutamax (Gibco). Cells were maintained in a humidified 37 °C incubator with a 5% CO₂ atmosphere. Aliquots of inhibitor (stock in 95% ethanol) were delivered onto 100 mm tissue culture plates, and the ethanol was evaporated before cells were seeded onto the plate. The cells were incubated at 37 °C for 24 h at which time they reached ~80% confluence. The cells were washed twice with DPBS (Gibco), removed from the plates by trituration and pelleted (800×g, 10 min). Cells were gently washed again with cold DPBS, (10 ml), and pelleted. The cells could be frozen at -80 °C at this point. Control cultures without inhibitor were treated in the same manner.

4.2.3. Western blot analyses

Frozen cells pellets were thawed on ice, and lysed using cell lysis buffer (1 ml of 50 mM Tris, pH 8.0, containing 150 mM NaCl, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 1% Triton X-100, 0.5% sodium deoxycholate) and by inversion rotation at 4 °C for 20 min. Cell debris were removed by centrifugation at 14,000 rpm in an Eppendorf 5430R microcentrifuge for 20 min at 4 °C. The cell lysates were then either used immediately or aliquoted and stored at -80 °C until required. SDS-PAGE loading buffer was added to an aliquot of each sample, and after heating at 95 °C for 10 min, aliquots were loaded onto 10% Tris·HCl polyacrylamide gels. After electrophoresis, the samples were electroblotted to nitrocellulose membrane (0.45 μ m, Bio-Rad). The membranes were blocked using 2% BSA (fraction V, Sigma) in PBST (PBS pH 7.4, 0.1% [v/v] Tween-20) and then incubated with blocking buffer containing the appropriate primary antibody, washed with PBST and then incubated with a horseradish peroxidase (HRP) conjugated secondary antibody. The membranes were washed again and detection of secondary HRP conjugate was done using a SuperSignal West Pico chemiluminescent detection kit (Pierce) and Chemidoc MP Imaging system (Biorad) (software Image Lab 5.2). For detection of O-GlcNAc modified proteins, mouse anti-O-GlcNAc IgM (clone CTD 110.6, BioLegend) was used as the primary antibody at a dilution of 1:2500, and goat anti-mouse-IgM-HRP conjugate (Santa Cruz Biotechnology) was used as the secondary antibody at a dilution of 1:2500. For detection of β -actin levels, mouse monoclonal anti- β -actin IgG (clone AC-40, Sigma) was used as the primary antibody at a dilution of 1:4000, and rabbit anti-mouse-IgG-HRP conjugate (Jackson ImmunoResearch) was used as the secondary antibody at a dilution of 1:10,000.

4.3. Synthesis

4.3.1. Ethyl (3*S*,4*R*,5*R*,6*R*)-4-acetamido-5,6-bis(benzyloxy)-3-((tert-butylidimethylsilyl)oxy) cyclohex-1-ene-1-carboxylate (**6**)

The enol triflate **5** (1052 mg, 1.67 mmol), Pd(OAc)₂ (75 mg,

0.33 mmol), PPh₃ (177 mg, 0.67 mmol), Et₃N (341 mg, 3.34 mmol), DMF (20 mL), and EtOH (10 mL) were added to a Schlenk tube, and the mixture was degassed by bubbling nitrogen gas for 10 min and bubbling CO gas for another 10 min. The reaction was stirred under CO atmosphere (1 atm) at 65 °C for 2 h. The reaction was cooled to RT, quenched with H₂O (50 mL), extracted with EtOAc (125 mL x 2), the combined organic layers were washed with brine (50 mL x 2), dried over MgSO₄ and concentrated under reduced pressure to afford a brownish oil. The residue was purified by column chromatography (SiO₂), eluted with pentane/Et₂O 2/1 to 1/1 to afford the ethyl ester **6** (627 mg, 1.13 mmol, 68% yield) as a colourless oil. **TLC** (pentane/Et₂O = 1/1), R_f = 0.4. **¹H NMR** (400 MHz, CDCl₃): δ = 7.43–7.23 (m, 10H), 6.75 (dt, J = 2.4, 0.9 Hz, 1H), 6.00 (d, J = 8.8 Hz, 1H), 4.68–4.54 (m, 5H), 4.51 (d, J = 10.5 Hz, 1H), 4.32–4.21 (m, 3H), 4.04 (dd, J = 4.7, 2.7 Hz, 1H), 1.77 (s, 3H), 1.32 (t, J = 7.1 Hz, 3H), 0.88 (s, 9H), 0.12 (s, 3H), 0.08 (s, 3H); **¹³C NMR** (100 MHz, CDCl₃): δ = 169.95, 165.64, 141.52, 137.64, 129.91, 129.55, 128.57, 128.40, 128.21, 128.04, 127.98, 127.86, 123.48, 77.20, 74.46, 73.82, 72.69, 72.43, 65.57, 60.93, 48.13, 25.74, 23.29, 18.11, 14.25, -4.96, -5.06; **IR** (ATR): ν = 3426, 2954, 2857, 2830, 1720, 1682, 1521, 1381, 1249, 1065, 838, 698 cm⁻¹; **ESI-MS (ES⁺)** found 554.2934 (M + H⁺), calc 554.2932 (M + H⁺); $[\alpha]_D^{25}$ = +11.87 (c = 1.0, CHCl₃).

4.3.2. Ethyl (3*S*,4*S*,5*R*,6*R*)-4-acetamido-5,6-bis(benzyloxy)-3-hydroxycyclohex-1-ene-1-carboxylate (**7**)

To a solution of the TBS protected ester **6** (626 mg, 1.13 mmol) in THF (25 mL, dry), a TBAF solution (1.36 mL, 1.0 M in THF, 1.36 mmol) was added at 0 °C. The solution was warmed to RT and stirred for 2 h. The solvent was evaporated under reduced pressure and the residue was purified by column chromatography, (SiO₂) eluted with EtOAc to afford the alcohol **7** (371 mg, 0.84 mmol, 74% yield) as a white solid. **TLC** (100% EtOAc), R_f = 0.38. **¹H NMR** (400 MHz, CDCl₃): δ 7.39–7.26 (m, 10H), 7.07 (d, J = 7.9 Hz, 1H), 6.94 (t, J = 1.7 Hz, 1H), 4.78–4.72 (m, 2H), 4.72–4.64 (m, 1H), 4.62–4.50 (m, 3H), 4.39–4.33 (m, 1H), 4.32–4.19 (m, 2H), 3.84 (dd, J = 4.0, 2.2 Hz, 1H), 1.81 (s, 3H), 1.32 (t, J = 7.1 Hz, 3H); **¹³C NMR** (100 MHz, CDCl₃): δ = 172.56, 165.59, 141.70, 137.26, 137.09, 128.61, 128.59, 128.31, 128.23, 128.19, 128.15, 127.69, 77.2, 74.16, 74.10, 72.09, 71.95, 67.23, 60.97, 48.74, 23.10, 14.21; **IR** (ATR): ν = 3379, 2901, 1717, 1657, 1543, 1248, 1073, 749, 699 cm⁻¹; **ESI-MS (ES⁺)** found 440.2065 (M + H⁺), calc 440.2068 (M + H⁺); $[\alpha]_D^{25}$ = +32.5 (c = 0.5, CHCl₃).

4.3.3. (3*S*,4*S*,5*R*,6*R*)-4-acetamido-3,5,6-trihydroxycyclohex-1-ene-1-carboxylic acid (**8**)

General Procedure 1: Step 1: To a solution of the ester **7** (15 mg, 34 μ mol) and pentamethylbenzene (10 mg, 68 μ mol) in dry CH₂Cl₂ (0.5 mL), BCl₃ (273 μ L, 273 μ mol, 1.0 M in CH₂Cl₂) was added at -78 °C and the reaction was stirred at -78 °C for 1 h. MeOH (1 mL) was added to quench the reaction, then the solvent was removed under reduced pressure. The residue was dissolved in H₂O (2.0 mL), washed with pentane (2.0 mL x 3), the aq. layer was concentrated under reduced pressure and the residue was used in the next step without further purification. Step 2: To a solution of the triol intermediate (6.7 mg) in H₂O (0.5 mL), a KOH solution (97 μ L, 1.0 M, 97 μ mol) was added at RT. The reaction was stirred for 1 h, neutralized to pH = 5 using Amberlyst 15 resin, filtered through a short pad of celite and the compound was washed with EtOH. The filtrate was concentrated under reduced pressure and purified by a preparative HPLC system equipped with a RP-HPLC column (Syn-ergi hydro, Phenomenex) to obtain the carboxylic acid **8** (4.1 mg, 18 μ mol, 53% yield over 2 steps) as a white solid. **M.p.**: 205.5–206.6 °C. **¹H NMR** (500 MHz, D₂O): δ 6.39 (d, J = 5.2 Hz, 1H), 4.39 (d, J = 7.7 Hz, 1H), 4.33 (t, J = 4.7 Hz, 1H), 3.97 (dd, J = 11.3,

4.1 Hz, 1H), 3.78 (dd, $J = 11.3, 7.6$ Hz, 1H), 2.08 (s, 3H); **^{13}C NMR** (125 MHz, $\text{D}_2\text{O} + \text{CD}_3\text{OD}$): $\delta = 175.83, 175.57, 141.04, 130.82, 73.72, 71.32, 66.08, 53.86, 23.45$; **IR** (ATR): $\nu = 3299, 1650, 1572, 1376\text{ cm}^{-1}$; **ESI-MS** (ES^-) found 230.0668 ($\text{M}-\text{H}^+$), calc 230.0670 ($\text{M}-\text{H}^+$); $[\alpha]_{\text{D}}^{\text{RT}} = +58.2$ ($c = 2.16, \text{MeOH}$).

4.3.4. Ethyl (3*S*,4*R*,5*R*,6*R*)-4-acetamido-5,6-bis(benzyloxy)-3-((methylsulfonyl)oxy)cyclohex-1-ene-1-carboxylate (**9**)

To a cold (0°C) solution of the alcohol **7** (288 mg, 0.66 mmol) in CH_2Cl_2 (10 mL), MsCl (150 mg, 1.31 mmol), DMAP (16 mg, 0.13 mmol) and NEt_3 (370 μL , 2.62 mmol) were slowly added. The reaction was stirred at 0°C for 1 h and at RT for 1 h. The solvent was removed under reduced pressure and the residue was directly purified by column chromatography (SiO_2), eluted with Et_2O to afford the mesylate **9** (244 mg, 0.47 mmol, 71% yield). **TLC** (100% Et_2O), $R_f = 0.35$. **^1H NMR** (400 MHz, CDCl_3): 7.42–7.22 (m, 10H), 6.90–6.84 (m, 1H), 6.61 (d, $J = 9.4$ Hz, 1H), 5.63–5.54 (m, 1H), 4.91–4.80 (m, 1H), 4.71 (dd, $J = 10.7, 0.7$ Hz, 1H), 4.62–4.53 (m, 2H), 4.51 (d, $J = 10.7$ Hz, 1H), 4.39–4.33 (m, 1H), 4.34–4.21 (m, 2H), 3.85 (dd, $J = 4.2, 2.5$ Hz, 1H), 3.08 (s, 3H), 1.79 (s, 3H), 1.34 (t, $J = 7.1$ Hz, 3H); **^{13}C NMR** (100 MHz, CDCl_3): $\delta = 170.00, 164.70, 136.99, 136.85, 136.41, 131.69, 128.67, 128.58, 128.36, 128.29, 127.89, 77.20, 74.28, 74.23, 74.15, 72.40, 71.60, 61.35, 46.05, 38.71, 23.22, 14.17$; **IR** (ATR): $\nu = 3393, 2935, 1719, 1667, 1524, 1360, 1250, 1174, 961, 884, 741\text{ cm}^{-1}$; **ESI-MS** (ES^+) found 518.1842 ($\text{M} + \text{H}^+$), calc 518.1843 ($\text{M} + \text{H}^+$); $[\alpha]_{\text{D}}^{\text{RT}} = +32.26$ ($c = 1.0, \text{CHCl}_3$).

4.3.5. Ethyl (3*R*,4*S*,5*R*,6*R*)-4-acetamido-5,6-bis(benzyloxy)-3-hydroxycyclohex-1-ene-1-carboxylate (**10**)

To a solution of the protected mesylate **9** (36 mg, 70 μmol), 18-crown-6-ether (1.8 mg, 7 μmol) in DMF (2.0 mL) and KNO_2 (30 mg, 348 μmol) were added at RT. The reaction was stirred at RT for 16 h, then diluted with EtOAc (15 mL), and washed with brine (5 mL x 2), dried over MgSO_4 and concentrated under reduced pressure. The residue was purified by column chromatography (SiO_2), eluted with EtOAc to afford the alcohol **10** (18 mg, 40 μmol , 57% yield) as a white solid. **TLC** (100% EtOAc), $R_f = 0.31$. **^1H NMR** (400 MHz, CDCl_3): δ 7.42–7.22 (m, 10H), 7.11–7.05 (m, 1H), 6.57 (d, $J = 7.7$ Hz, 1H), 4.75 (d, $J = 10.7$ Hz, 1H), 4.64–4.54 (m, 3H), 4.51–4.45 (m, 2H), 4.37–4.19 (m, 2H), 4.16–4.07 (m, 1H), 3.90–3.83 (m, 1H), 3.25 (d, $J = 10.2$ Hz, 1H), 1.75 (s, 3H), 1.34 (t, $J = 7.1$ Hz, 3H); **^{13}C NMR** (100 MHz, CDCl_3): $\delta = 169.70, 166.11, 139.36, 137.29, 136.64, 128.93, 128.76, 128.61, 128.44, 128.30, 128.22, 128.04, 77.20, 74.24, 73.81, 72.60, 71.98, 67.14, 61.18, 49.90, 23.14, 14.21$; **IR** (ATR): $\nu = 3300, 2872, 1717, 1548, 1251, 1055, 738, 698\text{ cm}^{-1}$; **ESI-MS** (ES^+) found 440.2067 ($\text{M} + \text{H}^+$), calc 440.2068 ($\text{M} + \text{H}^+$); $[\alpha]_{\text{D}}^{\text{RT}} = -84.38$ ($c = 1.0, \text{CHCl}_3$).

4.3.6. (3*R*,4*S*,5*R*,6*R*)-4-acetamido-3,5,6-trihydroxycyclohex-1-ene-1-carboxylic acid (**11**)

This compound was synthesized from the alcohol **10** following general procedure 1 used for the compound **8**. The obtained yield was 52% over 2 steps starting from 10 mg of starting material. Isolated as a white solid; **m.p.**: 128.1–128.8 $^\circ\text{C}$; **^1H NMR** (500 MHz, D_2O): δ 6.69 (s, 1H), 4.56 (d, $J = 8.0$ Hz, 1H), 4.40 (dt, $J = 9.5, 2.5$ Hz, 1H), 3.91 (dd, $J = 11.3, 9.3$ Hz, 1H), 3.65 (dd, $J = 11.3, 7.7$ Hz, 1H), 2.08 (s, 2H); **^{13}C NMR** (125 MHz, $\text{D}_2\text{O} + \text{CD}_3\text{OD}$): $\delta = 176.49, 171.46, 140.67, 134.36, 74.75, 72.72, 70.71, 57.79, 23.68$; **IR** (ATR): $\nu = 3314, 1699, 1644, 1564, 1253\text{ cm}^{-1}$; **ESI-MS** (ES^+) found 232.0813 ($\text{M} + \text{H}^+$), calc 232.0816 ($\text{M} + \text{H}^+$); $[\alpha]_{\text{D}}^{\text{RT}} = +70.6$ ($c = 0.8, \text{MeOH}$).

4.3.7. Ethyl (3*R*,4*S*,5*R*,6*R*)-4-acetamido-3-azido-5,6-bis(benzyloxy)cyclohex-1-ene-1-carboxylate (**17**)

To a solution of the mesylate **9** (100 mg, 0.19 mmol) in acetone (4.0 mL), a solution of NaN_3 (50 mg (0.77 mmol) in water (1 mL))

was added at 0°C . The reaction was stirred at 0°C for 4 h, quenched with a sat. NaHCO_3 soln. (2.0 mL), the compounds were extracted with EtOAc (10 mL x 2). The organic layers were combined, washed with brine (10 mL), dried over MgSO_4 and concentrated under reduced pressure. The residue (88 mg) was used for the next step without further purification.

4.3.8. Ethyl (3*R*,4*S*,5*R*,6*R*)-4-acetamido-3-amino-5,6-bis(benzyloxy)cyclohex-1-ene-1-carboxylate (**12**)

To a solution of the azide **17** (12 mg) in THF (0.5 mL) with H_2O (30 μL), PPh_3 (20.5 mg 77 μmol) was added. The reaction was stirred under nitrogen at RT for 2 h and then mixture was purified by column chromatography (SiO_2), eluted with $\text{CH}_2\text{Cl}_2/\text{MeOH}$ 20/1 to 10/1 to afford the amine **12** (7 mg, 16 μmol 62% yield over 2 steps) as a yellowish oil. **TLC** ($\text{CH}_2\text{Cl}_2/\text{MeOH} = 10/1$), $R_f = 0.30$. **^1H NMR** (400 MHz, CDCl_3): δ 7.40–7.22 (m, 10H), 7.12 (dd, $J = 4.6, 1.2$ Hz, 1H), 6.87 (d, $J = 8.2$ Hz, 1H), 4.73 (d, $J = 10.7$ Hz, 1H), 4.64–4.51 (m, 3H), 4.48 (dd, $J = 2.3, 1.2$ Hz, 1H), 4.46–4.40 (m, 1H), 4.34–4.22 (m, 2H), 3.86 (dd, $J = 3.5, 2.3$ Hz, 1H), 3.39 (d, $J = 4.5$ Hz, 1H), 1.74 (s, 3H), 1.71 (s, 2H), 1.34 (t, $J = 7.1$ Hz, 3H); **^{13}C NMR** (100 MHz, CDCl_3): $\delta = 169.31, 166.39, 142.57, 137.42, 137.20, 128.60, 128.27, 128.25, 128.10, 127.70, 127.21, 77.20, 73.94, 73.52, 71.97, 71.51, 60.99, 51.15, 50.98, 23.21, 14.24$; **IR** (ATR): $\nu = 3380, 2903, 1715, 1659, 1526, 1370, 1252, 1068, 739, 699\text{ cm}^{-1}$; **ESI-MS** (ES^+) found 439.2229 ($\text{M} + \text{H}^+$), calc 439.2228 ($\text{M} + \text{H}^+$); $[\alpha]_{\text{D}}^{\text{RT}} = -79.49$ ($c = 1.25, \text{CHCl}_3$).

4.3.9. (3*R*,4*S*,5*R*,6*R*)-4-acetamido-3-amino-5,6-dihydroxycyclohex-1-ene-1-carboxylic acid (**13**)

This compound was synthesized from the amine **12** following general procedure 1 used for the compound **8**. The obtained yield was 94% over 2 steps starting from 10 mg of starting material. Isolated as a white solid, **m.p.**: 213.5–214.3 $^\circ\text{C}$; **^1H NMR** (500 MHz, D_2O): δ 6.21 (s, 1H), 4.54 (d, $J = 8.3$ Hz, 1H), 4.16–4.04 (m, 2H), 3.78–3.72 (m, 1H), 2.10 (s, 3H); **^{13}C NMR** (125 MHz, $\text{D}_2\text{O} + \text{CD}_3\text{OD}$): $\delta = 176.90, 174.40, 142.89, 126.39, 73.96, 73.06, 54.05, 53.78, 23.63$; **IR** (ATR): $\nu = 3262, 2982, 1563, 1374, 1054, 1033, 1016\text{ cm}^{-1}$; **ESI-MS** (ES^+) found 231.0976 ($\text{M} + \text{H}^+$), calc 231.0976 ($\text{M} + \text{H}^+$); $[\alpha]_{\text{D}}^{\text{RT}} = -11.2$ ($c = 0.8, \text{H}_2\text{O}$).

4.3.10. 1*R*,2*R*,3*S*,4*S*)-3-acetamido-6-(acetoxymethyl)cyclohex-5-ene-1,2,4-triyl triacetate (**16**)

A sealed tube was charged with kirkamide (20 mg, 0.092 mmol), sodium acetate (90.7 mg, 1.11 mmol) and acetic anhydride (2 mL). The reaction was stirred at 90°C for 2 h. After consumption of the starting material, the reaction was quenched with ice and sat. NaHCO_3 soln. (3 mL). The aqueous phase was extracted with ethyl acetate (15 mL x 2) and the combined organic phases were washed with brine (5 mL x 2), dried over Na_2SO_4 and evaporated under reduced pressure. The product **16** was obtained as a pale yellow oil (32 mg, 0.086 mmol 93% yield). **TLC** ($\text{CH}_2\text{Cl}_2/\text{MeOH} = 10/1$), $R_f = 0.5$; **^1H NMR** (400 MHz, CDCl_3): δ 6.10 (dt, $J = 5.5, 1.5$ Hz, 1H), 5.85–5.74 (m, 2H), 5.33–5.25 (m, 2H), 4.68 (dd, $J = 13.3, 1.2$ Hz, 1H), 4.53 (ddd, $J = 11.5, 9.4, 3.9$ Hz, 1H), 4.36 (dd, $J = 13.4, 1.0$ Hz, 1H), 2.11 (s, 3H), 2.06–2.03 (m, 9H), 1.94 (s, 3H); **^{13}C NMR** (101 MHz, CDCl_3): δ 171.66 (s), 170.28 (s), 169.82 (s), 169.73 (s), 169.65 (s), 137.15 (s), 124.51 (s), 70.96 (s), 69.99 (s), 67.94 (s), 62.53 (s), 48.92 (s), 23.09 (s), 20.91 (s), 20.75 (s), 20.64 (s), 20.57 (s); **IR** (ATR): $\nu = 3328, 2954, 1745, 1666, 1371, 1225, 1046\text{ cm}^{-1}$; **ESI-MS** (ES^+) found 386.1439 ($\text{M} + \text{H}^+$), calc 386.1451 ($\text{M} + \text{H}^+$); $[\alpha]_{\text{D}}^{\text{RT}} = +24.7$ ($c = 0.15, \text{CHCl}_3$).

Dedication

This paper is dedicated to Professor Nuno Maulide on receipt of the Tetrahedron Young Investigator Award.

Declaration of competing interest

There are no conflicts of interest to declare.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.tet.2020.131386>.

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